Collaborative Study of a Method and Sampling Techniques For Determining Yeast Count in Maple Sirup

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Techniques for sampling and a method for determining the yeast count in maple sirup, studied collaboratively in 1966, have been revised and again studied collaboratively in 18 laboratories. Statistical analysis of collaborators' results indicated that sirup samples shipped in a frozen condition gave more concordant interlaboratory yeast counts. More homogeneous cell suspensions were obtained by mixing the sirup samples warmed to 80°F with a nonaerating stirrer. The method is recommended for adoption as official, first action.

The collaborative study of a method for determining yeast count in maple sirup conducted in 1966 was repeated this year after certain major changes in the method. Analysis of the 1966 collaborators' data indicated that two factors contributed to the discordant results reported: (*a*) nonuniform growth occurred under the wide variety of uncontrolled storage (incubation) conditions while the samples were in transit, and (*b*) the mixing method used to prepare the suspension of yeast cells for subsampling to obtain collaborators' samples and the collaborators' sample for analysis did not produce a homogeneous mixture. New procedures have been developed and tested that should eliminate these problems; the revised method was subsequently tested collaboratively.

Sample Storage.—A new method for handling the yeast-sirup samples while in transit was investigated. The method was adapted from that used for shipping bull semen; in the method, the sample is shipped while frozen in a Dry Ice package.

To test the effect of freezing on the survival of yeast cells and on the reproducibility of the numbers of surviving cells, the following tests were made: Five 175 ml samples of yeast-contaminated sirup contained in 8 oz glass bottles were packed in pulverized Dry Ice in polystyrene containers ($8\frac{1}{2} \times 8\frac{1}{2} \times 8\frac{3}{4}$″ with 1″ thick walls). The recessed container covers were attached with a rubber-silicone sealant to retard loss of the CO_2. Five samples with an original yeast count of 1.2×10^{-6} were kept frozen for 48 hr in the Dry Ice container and then transferred to a deep freeze for 5 days. At the end of the holding period the samples were thawed and yeast counts made. The yeast counts (cells/ml $\times 10^4$) after freezing were 2.5, 2.6, 2.6, 2.5, and 2.4; per cent kill ranged from 97.84 to 98.00; average $97.00 \pm 0.083 \times 10^4$; coefficient of variation 3.29. These results show that freezing caused a loss of 97.9% of the original yeast cells, but

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the numbers of remaining viable cells in the test samples showed a high reproducibility.

Investigation of improved methods of transportation disclosed that air lines could insure delivery within 24 hours at a point near to or at the location of each collaborators' laboratories. The shipments would be made via air freight on the air line serving that area. The 24 hours in transit would insure that the inoculated sirup would arrive at the destination while still frozen. The shipping date was selected so that the samples would reach the collaborators' laboratories on a work day, and would thus avoid storage at shippers' terminals over a weekend. All samples were packed at the same time, were taken directly to the air terminal, and were in transit by 9:00 P.M. of that date. Collaborators were instructed to keep the samples frozen until the day of the test, i.e., 6 days after the shipping date. This insured that all samples would be stored under the same conditions and for the same length of time. Thus, the number of viable yeast cells surviving the freezing process in each collaborator's sample would be the same.

Sample Preparation.—The nonconcordant results of the 1966 study (1) also indicated that the collaborators' sample for analysis lacked uniformity of yeast cell dispersion; the mixing technique used did not provide a homogeneous cell suspension. Subsequent studies made with laboratory mixers showed that at the high speeds required for dispersion of yeast cells in sirup, large but nonuniform amounts of air were whipped into the sample so that the measured volume of sample taken for dilution contained different amounts of sirup and/or yeast cells. To overcome this source of error, a new type of nonaerating stirrer made by Kraft Apparatus Company (no endorsement implied) was tested. With this stirrer, it was possible to attain high speeds and also produce uniform cell dispersion without incorporation of air bubbles in the sample. The effectiveness of the stirrer was enhanced by heating the sirup to 80°F (26.7°C). This temperature lowered the viscosity of the sirup to 91 centipoises, compared to 164 at room temperature, and gave the maximum lowering of viscosity without danger of destroying the yeast cells. This effect was demonstrated by analysis of two aliquots of an inoculated sirup, one mixed at room temperature (68°F) and the other at 80°F. Yeast counts (cells/ml $\times 10^{-6}$) of 5 replicate analyses at 68 and 80°F were 0.6–2.0 and 0.95–1.27, respectively; mean for each was 1.58 ± 0.567 and 1.09 ± 0.119, respectively; coefficient of variation was 35.92 and 10.89%, respectively. Results at these mixing temperatures show that samples mixed at 80°F gave more precise results and had a coefficient of variation less than one-third that for samples mixed at room temperature.

Based on these findings, a revised method is described for determining the number of yeast cells in maple sirup.

METHOD

Apparatus

(a) *Water bath.*—Constant temp., capable of holding H_2O temp. at $27 \pm 1°$.

(b) *Electric stirrer.*—Non-aerating (Kraft Apparatus, Inc., 125-19 Liberty Ave., Richmond Hill, N.Y. 11419, Model S-25-25A, or equiv.).

(c) *Beakers.*—Tall-form, 300 and 500 ml. Cover with Al foil before sterilizing.

(d) *Hypodermic syringe.*—5 ml (Luer-Lok), or equiv. with hypodermic needle, 14 gauge (Luer-Lok), 2″ long.

(e) *Dilution bottles.*—160 ml, 45×140 mm.

(f) *Pipets, serological.*—To deliver 1.0 ml with 0.1 ml graduations.

(g) *Petri dishes.*—100×15 mm.

Apparatus (c), (d), (f), and (g) are sterilized 1 hr in hot air oven at 160°; however, items (f) and (g) do not have to be sterilized when obtained as "single use" sterile plastic.

Culture Medium

Wort agar culture medium.—Boil 15.00 g malt ext., 0.78 g peptone, 2.75 g dextrin, 2.35 g glycerol, 1.00 g K_2HPO_4, 1.00 g NH_4Cl, 12.75 g maltose, and 20.00 g agar in 1 L H_2O until dissolved. Sterilize by autoclaving 15 min at 15 lb pressure.

Diagnostic Reagents

(a) *Phosphate buffer stock soln.*—pH 7.2, 0.25*M*. *See* 5.023(e).

(b) *Phosphate buffer dilution water.*—*See* 5.023(f).

(c) *Hypochlorite germicide soln.*—200 ppm available Cl. Add 5 ml 5.25% NaClO soln to 1 gal. H_2O.

Preparation of Sample Culture

Sterilize stirrer head by submerging in 400 ml germicide soln in 500 ml tall-form beaker for not $<$ 10 min. Rinse by immersing in three 450 ml portions sterile H_2O in 500 ml tall-form beakers.

Warm bottle contg sirup to 80°F in H_2O bath set at 80 $\pm$ 1.0°F. Transfer sirup to 300 ml sterile tall-form beaker, place in 80°F constant temp. H_2O bath, and insert sterilized stirrer. Position stirrer near bottom of beaker and off center to prevent forming vortex. Cover beaker with Al foil while stirring. Stir sirup 10 min at ca 500 rpm. If gas bubbles form, let sirup stand in H_2O bath until gas bubbles rise to surface.

Assemble sterile 14 gauge needle and 5 ml syringe. Remove cover from sirup sample and with needle held at least 1″ below surface of sirup, slowly draw 5.5–6.0 ml sirup into syringe. Invert syringe, holding needle vertically. Wipe excess sirup from needle with gauze pad wetted with alcohol. Holding pad around tip of needle, bring syringe plunger exactly to 5.0 ml graduation, expelling excess sirup and any air bubbles into sterile pad.

Make 10^{-1} diln of sirup by expelling 5 ml sirup completely from syringe into 45 ml sterile phosphate buffer diln blank, (b). Shake inoculated diln blank vigorously 10 sec, transfer 1 ml to petri dish with 1 ml pipet for 10^{-1} diln plate, and transfer 0.1 ml 10^{-1} diln with 1 ml pipet to petri dish for 10^{-2} diln plate. Transfer 1 ml of 10^{-1} diln to 99 ml sterile diln blank, (b), for 10^{-3} diln. Prep. 10^{-3} and 10^{-4} diln plates by using 1 and 0.1 ml vol. 10^{-3} sirup diln, resp. Transfer 1 ml 10^{-3} diln to 99 ml sterile diln blank, (b), to make 10^{-5} sirup diln. Prep. 10^{-5} and 10^{-6} diln plates by using 1 and 0.1 ml 10^{-5} diln, resp.

Pour 10–12 ml liquefied wort agar at 42–44° into each plate and mix with dild culture. After agar has solidified, invert plates and incubate 5 days at 21–25°. Count plates on 5th day, using Quebec Colony Counter, or equiv.

Results and Recommendation

This revised method and sampling technique were tested for precision and reliability before the collaborative test by the techniques recommended by Youden (2). Results of these tests on ten replicated analyses were concordant: standard deviation(s) of 0.74×10^6 from a mean cell count of 11.1×10^6 and a coefficient of variation of 6.62%.

The new shipping method guaranteed more rapid and direct delivery of sample to the collaborators. Although the shipment of samples frozen in Dry Ice results in a loss of about 98% of the viable yeast cells, it is preferable to shipment under uncontrolled conditions. Moreover, the use of a sufficiently heavy inoculum yields enough viable organisms after freezing to provide valid cell counts.

Hypodermic syringes are preferable to pipetting and weighing techniques for subsampling viscous maple sirup (3). The exact and replicate amounts of the sirup obtained make the method well suited for comparing yeast cell counts.

The nonaerating stirrer that was used for dispersing yeast cells in sirup warmed to 80°F provided a uniform cell suspension in the sirup without causing an aeration problem.

Collaborative Study.—The modified method and shipping procedure were tested in a collaborative study on two samples of maple sirup inoculated with a slow growing and facultative osmophilic saccharomyces yeast that was isolated from a naturally contaminated maple sirup. Sample I, with a low yeast count of 2.0×10^6 cells per ml, was prepared by inoculating sterile sirup 2 weeks before the collaborators' subsamples were taken. Sample II, with a higher yeast count of 4.3×10^6 cells per ml, was prepared by inoculating sterile sirup 7 weeks before the collaborators' subsamples were taken.

Eighteen microbiologists in as many laboratories participated in this study. To minimize the work, only a single sample was sent to each collaborator; nine collaborators were sent Sample I, and nine were sent Sample II. Collaborators were requested to make a minimum of five separate and complete determinations including sampling, plating, and counting. They were also requested to indicate the type of stirrer used in preparing the

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cell suspension for subsampling in their reports; they were cautioned not to whip air into the sirup if a nonaerating stirrer was used.

Collaborative Results.—Results obtained by the collaborators are given in Table 1; data from four and seven collaborators are tabulated for Sample I and Sample II, respectively. Seven samples were reported lost for the following reasons: one shipping container was crushed in transit; one sample bottle was broken in transit; another sample bottle was broken in the laboratory; one sample bottle broke while under laboratory refrigeration; one sample was overheated in the water bath and thus the yeast count was reduced to zero; in one laboratory the culture medium used would not solidify, and the collaborator believed this was due to the use of a very old culture medium; and one collaborator reported no growth but offered no explanation. The four losses due to breakage of the sample bottles suggest the use of plastic sample bottles.

The interlaboratory results for Sample I and Sample II (Table 1) show considerable variation as indicated by the mean ($\bar{x}$) of the collaborators' yeast counts. The coefficient of variation (v, %), which evaluates intralaboratory standard deviations (s) on a comparable basis, shows a definite grouping. With one exception, the collaborators who used nonaerating stirrers had low coefficients of variation of 4.01 and 14.21% for Sample I and 3.47 and 16.45% for Sample II, whereas those who used other types of mixers had (with one exception) much higher coefficients of variation: 31.63 and 42.11% for Sample I and 21.52 and 26.81% for Sample II. Of the two collaborators whose results did not agree with the general grouping, one had a very low coefficient of variation (3.53%) for a sample mixed with a laboratory mixer, whereas the other had a very high coefficient of variation (29.31%) for a sample mixed with a nonaerating stirrer. These results indicate that the nonaerating stirrer produced a satisfactory and homogeneous cell suspension and that other types of mixers are not suited for this purpose.

It is apparent from these data that heating sirup to 80°F during mixing and the use of mechanical devices for mixing have increased intralaboratory precision when compared to data obtained from the 1966 study where coefficients of variation ranged from 3.3 to 115.6%. In the 1967 study, only two collaborators had coefficients of variation exceeding 30%; neither collaborator used a nonaerating stirrer.

The lethal effect of freezing on the yeast populations in the collaborators' samples was, as anticipated, strong. However, counts reported by collaborators in this study ranged

Table 1. Summary of collaborative yeast counts for two maple sirup samples, 5 determinations/collaborator[a]

Coll.	Range, cells/ml × 10^{-3} Low	High	$\bar{x} \times 10^{-3}$	$s \times 10^{-3}$	v, %
		Sample I			
1	1.0	2.5	1.8	0.76	42.11
2	13.0	31.0	22.0	6.96	31.63
3[b]	2.2	3.0	2.6	0.36	14.21
4[b]	30.0	33.0	31.4	1.26	4.01
		Sample II			
5	9.0	19.0	13.8	3.7	26.81
6	19.0	21.0	20.0	0.71	3.53
7[b]	20.0	30.0	23.8	3.89	16.34
8[b]	25.0	34.0	30.3	3.15	10.40
9[b]	37.0	40.0	38.6	1.34	3.47
10	55.0	91.0	72.2	15.54	21.52
11[b]	50.0	120.0	88.6	25.97	29.31

[a] $\bar{x}$ = mean of determinations; s = standard deviation; v = coefficient of variation.
[b] Collaborator used nonaerating stirrer.

from 1.8×10^3 to 88.6×10^3 cells/ml, whereas counts reported in the 1966 study ranged from 1.0×10^3 to 2.3×10^6 cells/ml. Thus, samples sent to collaborators for the 1967 study had more uniform yeast populations than these submitted for study in 1966.

No collaborators raised any serious questions regarding the method. One noted that the method was "messy"; another felt that expelling the sirup from the syringe was awkward and required too much manual strength and dexterity.

The method is recommended for adoption as official, first action.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was adopted by the Association. See *This Journal* **51**, 401 (1968).

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